Exhibit D

Bioconjugate Chem. 2003, 14, 581−587

Synthesis of Linear Polyethylenimine Derivatives for DNA Transfection

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Received July 20, 2002; Revised Manuscript Received February 13, 2003

A series of linear polymers containing varying amounts of ethylenimine or *N*-propylethylenimine units were synthesized by hydrolysis and/or reduction of polyethyloxazolines. The pK_as of the polyamines were determined potentiometrically. Gel mobility shift assay showed that the efficiency of DNA complexation was related to the fraction of amino groups that are protonated at neutral pH. The effects of cationic charge density and molar weight of the polymers on the transfection efficiency were evaluated on HepG2 cells. The results obtained with different copolymers show that the transfection efficiency primarily depends on the fraction of ethylenimine units included in the polymer albeit the molar weight is also of importance. On the basis of the results obtained with poly(*N*-propylethylenimines), we also demonstrate that the high transfection efficiency of polyethylenimines does not solely rely on their capacity to capture protons which are transferred into the endo-lysosomes during acidification.

INTRODUCTION

The development in the past decade of vectors allowing delivery of genes into eukaryotic cells opened the possibility to treat genetic as well as acquired diseases by using DNA as a prodrug. Although nonviral vectors are significantly less efficient in mediating gene transfer than viral ones, they present several advantages such as ease of production and simplicity of handling. A large variety of cationic compounds, among them lipids, polymers, and peptides, were shown to be able to efficiently deliver nucleic acids into numerous cell lines. However, only a few of these vectors allow transgene expression after systemic administration of DNA complexes. One of the most powerful and versatile families of carriers are polyethylenimines (PEIs)[1] (*1*−*5*).

It was proposed that the high gene transfer efficiency of these polymers is due to their capacity to buffer endosomes (*1*). This hypothesis is based on the chemical structure of PEIs: they differ from other polymers such as polylysine in that only a fraction of the amino groups are protonated at physiological pH (*6, 7*). When the pH in the endo-lysosomal compartment becomes acidic, the capacity of PEIs to capture protons causes osmotic swelling and subsequent endosome disruption ("proton sponge effect"), thus permitting the release of endocytosed material into the cytosol. These findings have led to the design of new polymers that, like PEIs, exploit the acidification of the endocytic vesicles. A polylysine, partially substituted with histidyl residues which become cationic upon protonation of the imidazole groups at pH below 6.0, has a transfection efficiency that is significantly higher compared to nonmodified polylysine (*8*). These and other results (*9, 10*) support the idea that the buffering capacities of PEIs play an important role during transfection. However, since the exact mechanism of PEI-mediated transfection remains to be elucidated, it is possible that additional properties are required to obtain high transfection efficiencies.

We report here the synthesis of linear cationic polymers of various molar weight containing different proportions of ethylenimine units. Additionally, the synthesis of polymers containing *N*-propylethylenimine units was realized, allowing the study of the influence of the class of the amine function on the biological activity of the polymers.

Our work concerns the transfection efficiency of the different copolymers in relation to the fraction of ethylenimine units contained in the molecule. Moreover, we investigate whether the capacity of a polymer to capture protons in an acidic environment is sufficient to obtain a highly efficient transfection agent.

EXPERIMENTAL PROCEDURES

Materials. Methylene chloride, acetonitrile (sds), and 2-ethyl-2-oxazoline (Aldrich) were distilled over calcium hydride. Poly(2-ethyl-2-oxazoline) (50 kDa) (Aldrich), hydrochloric acid 37% (Aldrich), lithium aluminum chloride in THF 1 mol/L (Aldrich), methyl *p*-toluenesulfonate (Aldrich), and sodium hydroxide pellets (Aldrich) were used as received. The branched polyethylenimine of 25 kDa was from Aldrich. SMD2-LucDITR (7.6 kb) is an expression plasmid encoding the firefly luciferase gene under the control of the human cytomegalovirus (CMV) immediate-early promoter.

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[1] Abbreviations: PEI, polyethylenimine; BPEI, branched polyethylenimine; LPEI, linear polyethylenimine; EtOXZ, ethyloxazoline; PEtOXZ, polyethyloxazoline; LPNPEI, poly(*N*-propylethylenimine); LP(EtOXZ-*co*-EI), poly(ethyl-oxazoline-*co*-ethylenimine); LP(EtOXZ-*co*-NPEI), poly(ethyl-oxazoline-*co*-*N*-propylethylenimine); LP(EI-*co*-NPEI), poly(ethylenimine-*co*-*N*-propylethylenimine); LMW, low molar weight; DP_n, number polymerization degree.

10.1021/bc0200529 CCC: $25.00
Published on Web 05/01/2003

Polymer Characterization. Polymer analysis was performed by NMR spectroscopy (Brucker 200 or 300 MHz, solvents: $CDCl_3$ or D_2O); small angle neutron scattering spectroscopy (PACE (Orphée reactor, LLB Saclay), polymer concentration: 10 wt % in 1 M NaCl).

Synthesis of Poly(2-ethyl-2-oxazoline) (PEtOXZ) of 17.5 kDa. To a solution of methyl *p*-toluenesulfonate (74 mg, 397 mmol) in acetonitrile (60 mL) was added 8 mL of 2-EtOXZ (79 mmol). The reaction mixture was stirred refluxing for 143 h. After evaporation, the crude product was dissolved in methylene chloride and precipitated in diethyl ether. After filtration, the product was dried in vacuo yielding 7.05 g (88%) of a yellow powder. ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.1 (s, $NCOCH_2CH_3$); 2.3 (m, $NCOCH_2CH_3$); 3.4 (s, $N(COEt)CH_2CH_2$); 7.1 and 7.6 (d, H_{ar}).

Synthesis of a Linear Polyethylenimine (LPEI) of 7.3 kDa. One gram of poly(2-ethyl-2-oxazoline) ($M_{n(NMR)}$ = 17.5 kDa) was heated with a mixture of concentrated (37%) hydrochloric acid (11 mL) and water (8 mL) at 110°C for 3 h. After evaporation in vacuo of the solvent and the newly formed propionic acid, the product was dissolved in water, and NaOH pellets were added until pH 9−10 was reached. The aqueous layer was then evaporated and the residue washed with methylene chloride. After filtration, the organic layer was dried (Na_2SO_4) and evaporated. Yield was 320 mg (71%). ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.7 (br m, $NHCH_2CH_2$); 2.7 (s, $NHCH_2CH_2$).

Synthesis of Poly(ethyl-oxazoline-*co*-ethylenimine) Copolymers (LP(EtOXZ-*co*-EI)). As a typical example, a copolymer was prepared as follows: to 4 g of $PEtOXZ_{50kDa}$ (0.08 mmol) dissolved in 80 mL of water was added 2.4 mL of hydrochloric acid (29 mmol), and the mixture was heated under reflux for 67 h. The aqueous layer was then evaporated to remove the propionic acid. The residue was dissolved in water, and NaOH pellets were added for neutralization. After evaporation, the product was dissolved in methylene chloride. After filtration of sodium chloride and propionate residue, the organic layer was dried (Na_2SO_4) and evaporated in vacuo. Yield of copolymer was 2.83 g (96%), with a 60% hydrolysis content. ^{1}H NMR (D_2O): δ (ppm) = 1.1 (t, $NCOCH_2CH_3$); 2.4 (q, $NCOCH_2CH_3$); 2.8 (m, $NHCH_2CH_2$); 3.5 (m, $N(COEt)CH_2CH_2$).

Synthesis of Poly(ethyl-oxazoline-*co*-*N*-propylethylenimine) (LP(EtOXZ-*co*-NPEI)) and Poly(*N*-propylethylenimine) (LPNPEI). To a solution of poly(2-ethyl-2-oxazoline)$_{50kDa}$ (4 g, 0.08 mmol) in methylene chloride (400 mL) was added $LiAlH_4$ (17 mL, 17 mmol). The reaction mixture was stirred refluxing for 14 h. After addition of water, the organic layer was filtered to remove LiOH and $Al(OH)_3$ and then the solvent was evaporated. Yield was 2.16 g (58%) with a 64% reduction content for polymer. ^{1}H NMR ($CDCl_3$): δ (ppm) = 0.8 (t, $NCH_2CH_2CH_3$); 1.1 (t, $NCOCH_2CH_3$); 1.4 (q, $NCH_2CH_2CH_3$); 2.3 (t, $NCOCH_2CH_3$); 2.6 (m, $N(CH_2Et)CH_2CH_2$); 3.4 (m, $N(COEt)CH_2CH_2$).

For poly(*N*-propylethylenimine) synthesis, an excess of $LiAlH_4$ ([$LiAlH_4$]/[NCOEt] ≥ 2) solution was used to reduce 1 equiv of *N*-propionylethylenimine monomer unit of poly(2-ethyl-2-oxazoline).

Synthesis of Poly(ethylenimine-*co*-*N*-propylethylenimine) Copolymers (LP(EI-*co*-NPEI)). Typically, 4 g of $PEtOXZ_{50kDa}$ (0.08 mmol) was hydrolyzed with HCl (4.3 mL, 52 mmol) as described above. The product (2 g) was isolated and then reduced by $LiAlH_4$ (30 mL, 30 mmol). Yield was 1.94 g (93%). ^{1}H NMR (D_2O): δ (ppm) = 0.9 (t, $NCH_2CH_2CH_3$); 1.5 (m, $NCH_2CH_2CH_3$); 2.6 (m, $NCH_2CH_2CH_3$); 2.8 (m, $N(nPr)CH_2CH_2$); 3.0 (s, $NHCH_2CH_2$).

Potentiometric Titrations. To a solution of either $LPEI_{22kDa}$ (33 mg, 1.4 mmol), LP(EtOXZ$_{20}$-*co*-EI$_{270}$-*co*-NPEI$_{210}$) (22 mg, 0.7 mmol), or $LPNPEI_{42.5kDa}$ (45 mg, 1 μmol) in water was added HCl (10 mL, 1.0 10^{-1} mol/L). After stirring, potentiometric titration of solution was measured with 9.2 × 10^{-2} M NaOH as a titrant by using the automatic titration system LogipH. The pK_a values of monomer units, respectively *N*,*N*-diethylethylenediamine, *N*,*N*,*N*′,*N*′-tetraethylethylenediamine, and pentaethylene hexamine were also determined by direct titration with LogipH using 0.1 M HCl.

Cell Culture. Dulbecco's modified Eagle medium (DMEM; Gibco-BRL) was supplemented with 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% of fetal calf serum (FCS; HyClone). Human hepatocarcinoma cells (HepG2 cells; American Type Culture Collection) were used for the transfection experiments.

Preparation of Polycation/DNA Complexes. Four micrograms of plasmid DNA and the desired amount of polymer (1 or 0.5 mg/mL solution, depending on the polymer) were each diluted in 100 μL of 150 mM NaCl and gently mixed. After 15 min of incubation, the mixture was diluted with serum-free medium to a final volume of 1 mL.

DNA Retardation Assay. DNA binding was studied by means of an agarose gel retardation assay. Plasmid DNA (1 μg) and increasing amounts of polymer were each diluted in 25 μL of 150 mM NaCl and mixed. After 15 min, samples (20 μL) were electrophoresed through a 1% agarose gel using Tris-borate-EDTA buffer. The DNA was visualized after ethidium bromide staining.

Transfection Experiments. Cells plated in 24-well plates (Costar) were transfected once confluency reached 50−80%. For transfection, 0.5 mL of serum-free medium containing the DNA complexes were transferred into each well. After incubation for 2 h 30 to 4 h at 37°C, the medium was replaced with fresh one containing 10% FCS. Luciferase activity was measured 24−48 h after transfection. Each experiment was carried out several times; within a series, experiments were done in duplicate.

For luciferase activity, cells were harvested in 250 μL of lysis buffer (8 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 1% Triton X-100, 15% glycerol, and 25 mM Tris-phosphate buffer pH 7.8). The cell lysate was then transferred into Eppendorf tubes and centrifuged for 5 min at 10 000*g* to pellet debris. Luciferase light units were measured in a 96-well plate format with a PhL luminometer (Mediators Diagnostika) from an aliquot of the supernatant (50 μL). The measurement was done over 10 s after automatic injection of 100 μL of assay buffer (lysis buffer without Triton X-100 supplemented with 2 mM ATP) and 100 μL of luciferin solution (167 μM in water; Molecular Probes). Luciferase background (about 300 light units) was subtracted from each value, and the transfection efficiency was expressed as total light units/10 s/well (with 1 light unit = 10 counts) and are the means of duplicates. The protein content of the transfected cells was measured by Bradford dye-binding using the BioRad protein assay.

RESULTS AND DISCUSSION

Synthesis of Linear Polyethylenimines (LPEIs). High molar weight LPEI was obtained as described by hydrolysis of the commercial poly(2-ethyl-2-oxazoline)

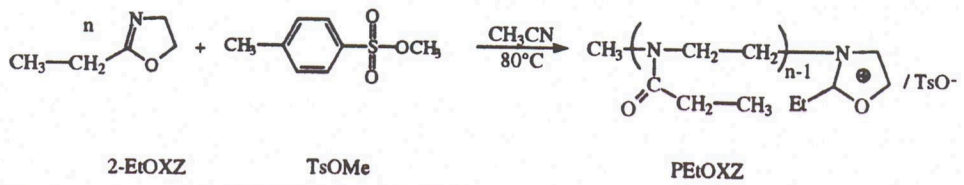

Figure 1. Polymerization scheme of 2-EtOXZ initiated by TsOMe.

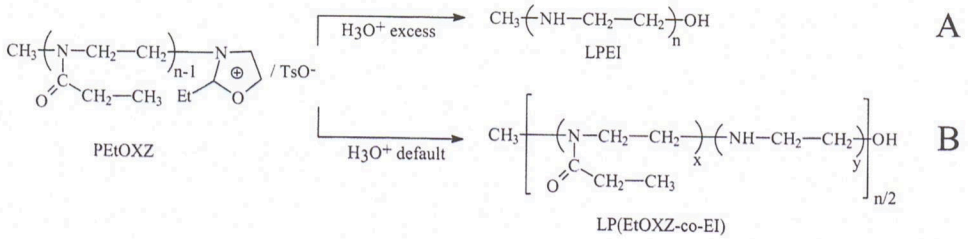

Figure 2. Scheme of acid hydrolysis of poly(2-ethyl-2-oxazoline) followed by neutralization (A) with an excess of HCl and (B) with a default of HCl.

Table 1. pK_a Values of the Polymers and Their Model Monomer Molecules

compd	TEDA[a]	DEDA[b]	PEHA[c]	LPNPEI 42.5 kDa	LPNPEI 170 kDa	LPEI 22 kDa	LP ($EtOXZ_{20}$-*co*-EI_{270}-*co*-$NPEI_{210}$)
pK_{a1}	6.2	6.9					
pK_{a2}	9.5	9.9					
pK_a [d]			8.2	7.2	7.2	7.9	7.7

[a] *N,N,N,N*-Tetraethylethylenediamine. [b] *N,N*-Diethylethylenediamine. [c] Pentaethylene hexamine. [d] Average pK_a values of the polymers.

(PEtOXZ) of 50 kDa in acidic aqueous medium under reflux (*11*). Oligomers of PEtOXZ, which are precursors of LPEIs of low molar mass, were synthesized by cationic polymerization of 2-ethyl-2-oxazoline (EtOXZ) initiated by methyl *p*-toluenesulfonate (TsOMe) in acetonitrile at 80°C, by varying the ratio $[EtOXZ]_0/[TsOMe]_0$ (Figure 1). After workup and purification, two PEtOXZ of different molar mass were collected and analyzed by ^{1}H NMR spectroscopy. The spectra indicate the presence of peaks corresponding to the amide group at 1.1, 2.3, and 3.4 ppm, and the aromatic proton peaks corresponding to the tosylate counteranion of the ionic terminations of the polymer at 7.1 and 7.6 ppm (*12*). The peaks corresponding to the ethyl group of the oxazolinium ion and that of the initiating methyl group are hidden by other signals. As it was previously reported, polymerization of EtOXZ initiated by TsOMe proceeds according to a living process, all chains being under oxazolinium tosylate ends (*12, 13*). Consequently, the number average polymerization degrees (DP_n) of polymers could be estimated by ^{1}H NMR spectroscopy from the determination of the ratio of the peak areas corresponding respectively to the propanoyle groups (1.1 ppm) to the aromatic part of tosylate ones (7.1 and 7.6 ppm). In these conditions, two PEtOXZ of 8 and 17.5 kDa were synthesized and characterized.

Acid hydrolysis of the poly(ethyl oxazoline)s was carried out using an excess of hydrochloric acid (Figure 2A). According to literature, the NMR spectra of the different LPEIs obtained (3.6, 7.3, and 22 kDa) confirmed the presence of methylene and NH groups by two peaks respectively at 2.7 and 1.7 ppm and the absence of signals corresponding to amide groups, showing thereby that the hydrolysis was complete (*11*). Of note, the disappearance of the signals corresponding to the tosylate counteranion of the precursors shows that the chain ends were hydrolyzed, producing a chain end of the type *N*-(2-hydroxyethyl)ethylenimine (Figure 2A). Neutron scattering of the protonated LPEI of 22 kDa showed that this polymer is a high molar mass rigid rod like structure, which is in tune with the repulsion effect of neighboring positive charges.

Potentiometric titration of this LPEI was then carried out. Neutral LPEI being insoluble in water, the titration requires the addition of an excess of hydrochloric acid and back-titration of this excess. The average pK_a is equal to 7.9 which shows that at a physiological pH more than 90% of the amino groups are protonated (Table 1). To check the relevancy of this pK_a determination, the titration of a model molecule, namely *N,N*-diethylethylenediamine, was also performed (Figure 3A). Two pH jumps can be seen on the curve corresponding to the two amino groups. This difference of basicity between two neighboring amino sites was already described in the literature (*14*). The protonation of one of the two sites on the same molecule influences the second one, thereby decreasing its basicity. The value of the acidity constants given by the pH at half equivalence (pK_{a1} = 9.9 and pK_{a2} = 6.9 respectively) allow to calculate an average pK_a of 8.4. The average pK_a of 7.9 of LPEI indicates a small decrease of the basicity of the amine functions in the polymer. This is due to the well-known polyelectrolyte effect and is in agreement with data reported in the literature (*15*).

Synthesis of Poly(2-ethyl-2-oxazoline-*co*-ethylenimine) Copolymers (LP(EtOXZ-*co*-EI)). Incomplete acid hydrolysis of commercial samples of $PEtOXZ_{50kDa}$ was carried out by varying the acid concentration in the reaction medium (Figure 2B). Different random copolymers LP(EtOXZ-*co*-EI) were obtained corresponding to various hydrolysis ratios with a yield of about 90%. The linear relationship obtained by plotting the hydrolysis ratio versus the initial acid concentration (Figure 4A) indicates a good control of the hydrolysis. This plot also suggests that there is a small initial hydrolysis even in the absence of hydrochloric acid.

The ^{1}H NMR spectra of the products collected after medium neutralization show the peaks of various methylene groups which correspond respectively to ethylen-

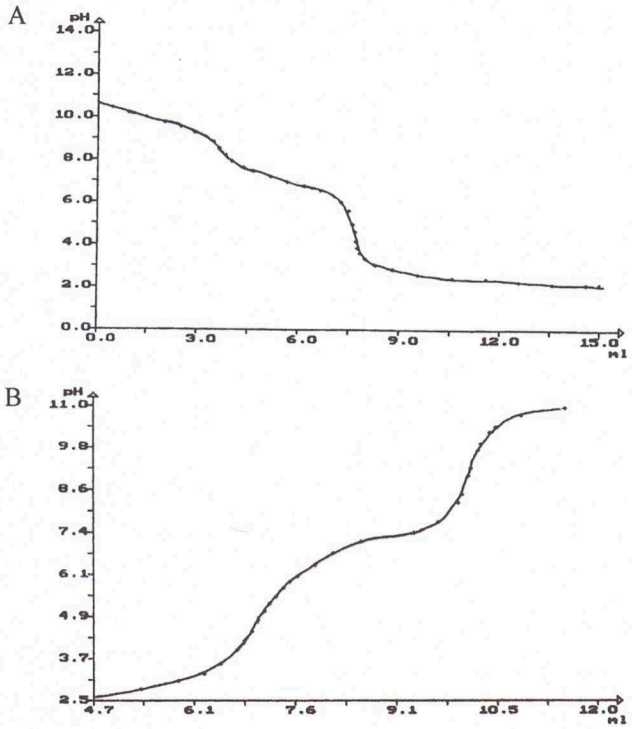

Figure 3. (A) Potentiometric titration of *N,N*-diethylethylenediamine. The titration was carried out by using 0.1 M HCl as titrant. (B) Potentiometric titration of acidic $LPNPEI_{42.5kDa}$ solution by using 9.2×10^{-2} M NaOH as titrant.

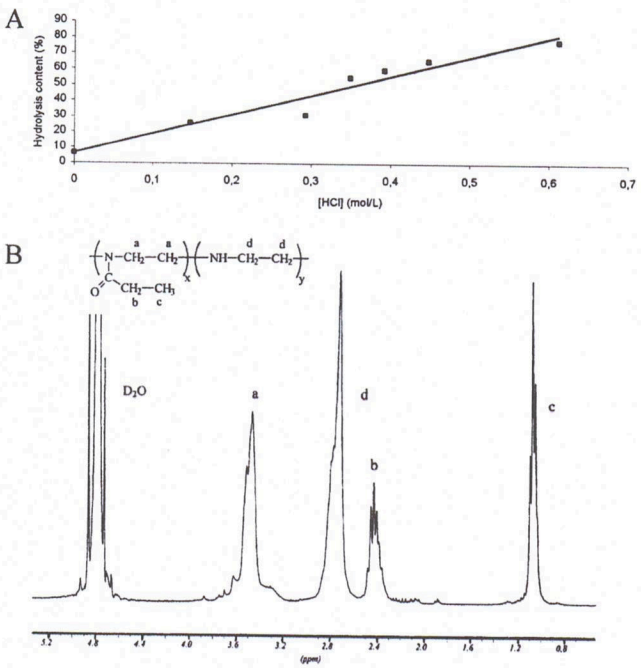

Figure 4. (A) Ethylenimine monomer unit content in copolymers obtained by partial hydrolysis of poly(2-ethyl-2-oxazoline) vs HCl concentration used. (B) ^{1}H NMR spectrum (300 MHz) of $LP(EtOXZ_{200}\text{-}co\text{-}EI_{300})$ in D_2O after neutralization.

imine units (2.8 ppm) and ethylenimine units substituted by the *N*-propanoyl side groups (3.5 ppm) (Figure 4B). The ratio of the areas of these peaks allowed the determination of the ethylenimine unit content in the different copolymers produced according to this method. However, in contrast to previously published results, no signals corresponding to propanoate residues were detected on the spectra (1.1 and 2.2 ppm) (*16*). This indicates, first, that the workup procedure applied at the

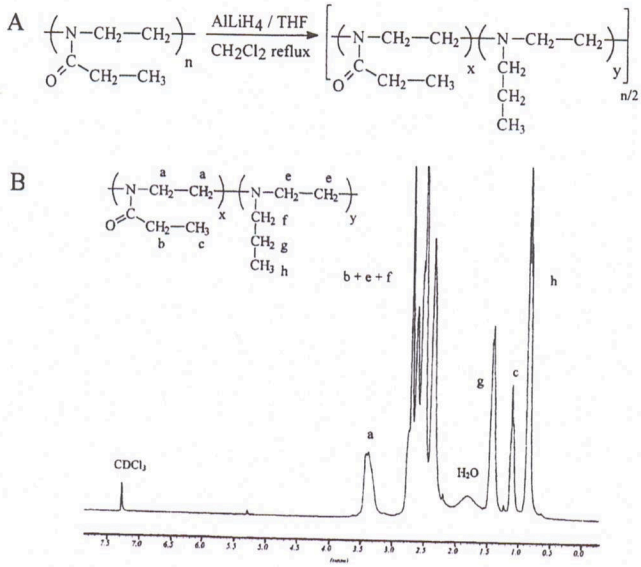

Figure 5. (A) Synthetic route of LP(EtOXZ-*co*-NPEI) and LPNPEI polymers by reduction of PEtOXZ using $LiAlH_4$. (B) ^{1}H NMR spectrum (300 MHz) of LP(EtOXZ-*co*-NPEI) with 81% reduction content in $CDCl_3$.

end of reaction, i.e., neutralization followed by dichloromethane extraction, is a more efficient method than that used by Jeong et al. to get rid of the sodium propanoate produced by the neutralization reaction. Second, it shows that the propanoate groups eventually present on NMR spectra cannot be attributed to NH_2^+/propanoate ion pairs (*16*).

Synthesis of Poly(2-ethyl-2-oxazoline-*co*-*N*-propylethylenimine) Copolymers (LP(EtOXZ-*co*-NPEI)). The hydrogenation of poly(2-methyl-2-oxazoline) giving poly(*N*-ethylethylenimine) by $LiAlH_4$ or AlH_3 has been previously reported in the literature (*17*). We applied this to poly(2-ethyl-2-oxazoline)s of different polymerization degrees by using various quantities of $LiAlH_4$ in order to obtain fully or partially reduced polymers (Figure 5A). This technique applied to $PEtOXZ_{50kDa}$ allowed us to obtain poly(2-ethyl-2-oxazoline-*co*-*N*-propylethylenimine) (LP(EtOXZ-*co*-NPEI)) copolymers with a good control of the hydrogenation ratio. On the other hand, by using an excess of hydride, PEtOXZ of different DP_n gave homopolymers LNPEIs with various molecular weights. ^{1}H NMR spectroscopic analysis of LP(EtOXZ-*co*-NPEI) samples obtained by incomplete reduction of $PEtOXZ_{50kDa}$ showed the decrease of the signals corresponding to the propanoyl groups and the presence of signals corresponding to newly formed *N*-propyl groups at 0.8, 1.4, and 2.6 ppm (Figure 5B). The disappearance of the peaks in the aromatic range corresponding to the tosylate counteranions of the ionic termini on the starting polymers confirmed that the oxazolinium cycle was reduced into a *N*-(ethyl)propylamine chain end. The reduction content was determined from the ratio of the surface of the methyl resonance ($CH_3CH_2CH_2N$) at 0.8 ppm to that of the total content of methyl groups of the polymer (0.8 + 1.1 ppm).

Potentiometric titration gave the average pK_a of the tertiary polyamine $LPNPEI_{42.5\ kDa}$ that was found equal to 7.2 (Figure 3B). This value which is lower than that of $LPEI_{22\ kDa}$ is in agreement with the different pK_a of the model molecule *N,N,N′,N′*-tetraethylethylenediamine which are respectively equal to 6.2 and 9.5, showing unambiguously that the tertiary amino sites are of lower basicity than the secondary ones (Table 1). Thus, LPN-

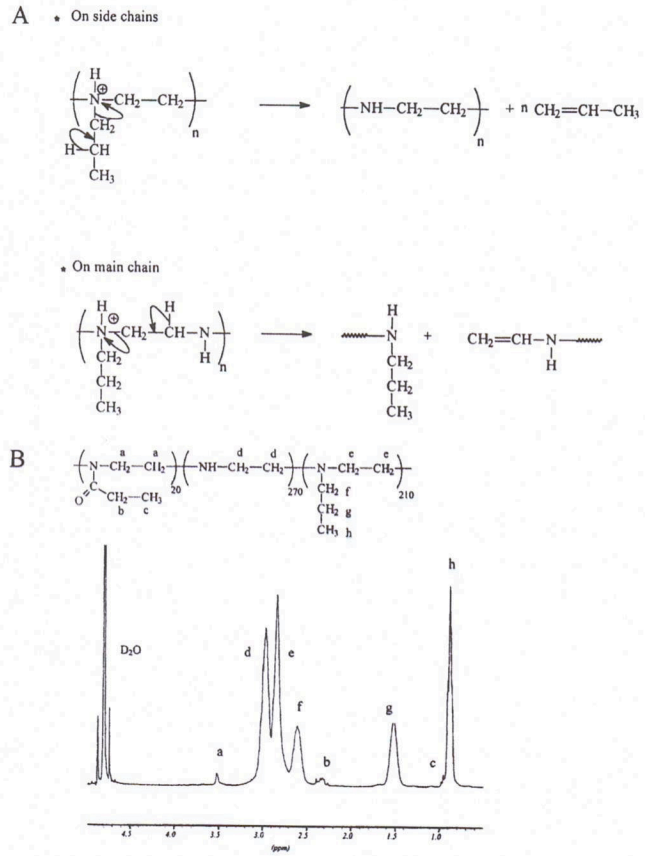

Figure 6. (A) Degradation process of LP(EtOXZ-*co*-NPEI) during acid hydrolysis. (B) ^{1}H NMR spectrum (300 MHz) of LP(EtOXZ$_{20}$-*co*-EI$_{270}$-*co*-NPEI$_{210}$) in D_2O.

PEI polymers are less protonated at a physiological pH (about 50%) than LPEIs.

Synthesis of Poly(ethylenimine-*co*-*N*-propylethylenimine) (LP(EI-*co*-NPEI)). The synthesis of poly(ethylenimine-*co*-*N*-propylethylenimine) (LP(EI-*co*-NPEI)) of different ratios using hydrolysis or reduction reactions was attempted starting from LP(EtOXZ-*co*-NPEI) and LP(EtOXZ-*co*-EI).

Hydrolysis was first carried out from LP(EtOXZ-*co*-NPEI) in hydrochloric medium under reflux for 18 h. ^{1}H NMR analysis of the residue obtained at the end of reaction indicated the absence of the characteristic *N*-propylethylenimine units, suggesting that some degradation occurred. A possible explanation could be found in the Hoffman degradation, inducing the cleavage of the *N*-propyl groups by β elimination (Figure 6A).

Starting again from the same copolymers LP(EtOXZ-*co*-NPEI) of various reduction ratios, hydrolysis in basic medium was tested, using different sodium hydroxide concentrations under reflux for different reaction times. Table 2 shows that in all cases and whatever the NPEI unit content of starting polymers, there always remained 20% of amide units, even with the highest sodium hydroxide concentration or the longest reaction time. This observation can be explained by some repulsion induced by the presence of tertiary amino groups on the nucleophilic reactant.

Since the two types of hydrolysis failed to give access to the copolymer LP(EI-*co*-NPEI), we tested whether the reduction of the amide units of the copolymers LP(EtOXZ-*co*-EI) having different hydrolysis ratios would allow to obtain the desired compounds. The results are presented in Table 3. The determination of the NPEI unit content of the products was done by ^{1}H NMR spectroscopy. Using an excess of reducing agent, this method gave a total reduction of the amide sites, whatever the fraction of ethyloxazoline units in the starting copolymer. The nearly complete reduction was shown by the presence of residual signals corresponding to the amide group and characteristic peaks of the secondary and tertiary ethylenimine units respectively at 3.0 and 2.8 ppm (Figure 6B).

Table 2. Hydrolysis of Poly(ethyl-oxazoline-*co*-*N*-propylethylenimine)s (LP(EtOXZ-*co*-NPEI)) in Basic Aqueous Solution

	starting polymer LP(EtOXZ-*co*-NPEI)[a]		
NPEI [b]content (%)	64	83	81
[NaOH]/[NCOEt] [c]	2	2.2	3.6
time (h)	17	17	67
EI [d]content (%)	17	0	0
resulting polymer composition	LP(EtOXZ$_{95}$-*co*-EI$_{85}$-*co*-NPEI$_{320}$) [e]	LP(EtOXZ$_{85}$-*co*-NPEI$_{415}$)	LP(EtOXZ$_{95}$-*co*-NPEI$_{405}$)

[a] Total number of monomer units = 500. [b] *N*-propylethylenimine monomer unit. [c] *N*-propanoylethylenimine monomer unit concentration. [d] Ethylenimine monomer unit. [e] Polymer obtained from precursor LP(EtOXZ$_{180}$-*co*-NPEI$_{320}$). This polymer was not tested in transfection experiments.

Table 3. Reduction of Poly(ethyl-oxazoline-*co*-ethylenimine)s (LP(EtOXZ-*co*-EI)) with $LiAlH_4$ in THF/CH_2Cl_2

	starting polymer LP(EtOXZ-*co*-EI) [a]		
EI[b]content (%)	26	54	76
[$LiAlH_4$]/[NCOEt] [c]	2.2	2.1	3.3
time (h)	7.5	16.5	16.5
yield (%)	51	72	68
NPEI [d]content (%)	56	42	24
resulting polymer composition	LP(EtOXZ$_{90}$-*co*-EI$_{130}$-*co*-NPEI$_{280}$)	LP(EtOXZ$_{20}$-*co*-EI$_{270}$-*co*-NPEI$_{210}$)	LP(EI$_{380}$-*co*-NPEI$_{120}$)

[a] Total number of monomer units = 500. [b] Ethylenimine monomer unit. [c] *N*-propanoylethylenimine monomer unit concentration. [d] *N*-propylethylenimine monomer unit.

Of note, the low downfield shift of the methylene protons (CH_2CH_2NH) of the ethylenimine units at 3.0 ppm of the copolymer LP(EI-*co*-NPEI), compared to that of the same protons of the copolymer LP(EtOXZ-*co*-EI) at 2.8 ppm, can be explained by the more electron-withdrawing effect of secondary amino groups in this case, due to the loss of hydrogen bonding between NH and propanoyl groups, which probably exists in the former copolymer.

The determination of the average pK_a of the copolymer LP(EtOXZ$_{20}$-*co*-EI$_{270}$-*co*-NPEI$_{210}$) is in tune with the weaker basicity of the tertiary amines (Table 1).

DNA Retardation Assay. The polymers were first tested for their capacity to interact with plasmid DNA. Therefore, increasing amounts of polymer were mixed with a constant amount of DNA, and the complexes were electrophoresed through an agarose gel. In this system, the polymer/DNA interactions are visualized by the retardation of the migration of DNA. As shown in Table 4, all the polymers except PEtOXZ were able to complex DNA. The LPEIs and the LP(EtOXZ$_{120}$-*co*-EI$_{380}$) copolymer were the most efficient in retarding DNA migration whereas the LPNPEIs of low molar weight and the LP(EtOXZ$_{350}$-*co*-EI$_{150}$) copolymer were the least efficient. It is interesting to note that homopolymers containing *N*-propylethylenimine units are weaker complexing agents than random LP(EtOXZ-*co*-NPEI) copolymers. The more efficient DNA complexation observed with the latter polymers may be due to the fact that the amino sites are partially isolated from each others by amide units, thereby increasing their basicity (see Table 1 for pK_a values). Moreover, our results show that the complex-

Table 4. Efficiency of the Different Polymers

polymer	DNA retardation (μg polymer)[a]	transfection efficiency[b]
LPEI (MW = 3600)	0.5	100
LPEI (MW = 7300)	0.5	500
LPEI (MW = 22000)	0.5	1000
LPNPEI (MW = 6100)	>4	0.1 <
LPNPEI (MW = 14400)	>4	0.1 <
LPNPEI (MW = 42500)	2.5	0.2
LPNPEI (MW = 171700)	2	1
LP(EI_{380}-*co*-$NPEI_{120}$) (MW = 26600)	1	30
PEtOXZ (MW = 50000)	no retardation	-
LP($EtOXZ_{420}$-*co*-$NPEI_{80}$) (MW = 48900)	2	55
LP($EtOXZ_{90}$-*co*-$NPEI_{90}$) (MW = 16300)	2	50
LP($EtOXZ_{180}$-*co*-$NPEI_{320}$) (MW = 45500)	1	3
LP($EtOXZ_{350}$-*co*-EI_{150}) (MW = 41500)	15	0.2
LP($EtOXZ_{200}$-*co*-EI_{300}) (MW = 32700)	1	55
LP($EtOXZ_{120}$-*co*-EI_{380}) (MW = 28900)	0.5	450
LP($EtOXZ_{90}$-*co*-EI_{130}-*co*-$NPEI_{280}$) (MW = 38800)	2.5	0.2
LP($EtOXZ_{20}$-*co*-EI_{270}-*co*-$NPEI_{210}$) (MW = 31500)	2.5	150

[a] Approximative amount of polymer (μg) needed for complete retardation of 1 μg of DNA. [b] The activity of the polymers was evaluated on HepG2 cells by using the reporter gene luciferase. The value 1000 was given to the transfection efficiency of the LPEI of 22 kDa.

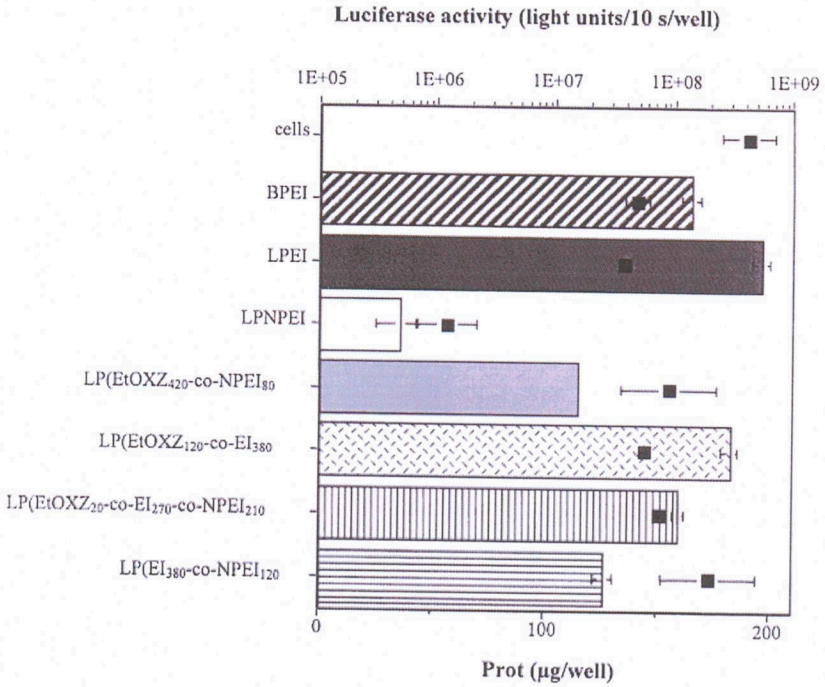

Figure 7. Evaluation of the transfection efficiency of PEI derivatives on HepG2 cells. Increasing amounts of polymer were mixed with a constant amount of reporter gene (4 μg per duplicate). The complexes were incubated in serum-free culture medium for 3 h with HepG2 cells. The luciferase activity was measured 30 h thereafter. The transfection efficiency (bars) was expressed as total light units/10 s/well and is the mean of the duplicates. Only the optimal condition for each compound is shown. The protein content (filled squares) was determined by using the BioRad protein assay.

ation efficiency increased with an increase of the fraction of the secondary amine functions and consequently with the decrease of the proportion of tertiary amines.

Taken together, these results show that the efficiency of complexation is related to the fraction of amino groups that are protonated at neutral pH. Thus, the higher the pK_a of the amines, the more efficient is the neutralization of the phosphate groups of the DNA.

Evaluation of the Transfection Activity. The transfection activity of the different polymer series was tested on human hepatocarcinoma cells (HepG2). Increasing amounts of polymer were complexed with 4 μg of a plasmid encoding a luciferase reporter gene under the control of the human cytomegalovirus (CMV) immediate-early promoter and applied to the cells for 3 h in the absence of serum. The luciferase activity and the protein content were measured 1 or 2 days thereafter. For comparison, we included in our assays the branched PEI of 25 kDa (BPEI) and the linear PEI of 22 kDa (LPEI), both of which were shown to be among the most efficient in vitro transfection agents (*1*, *2*, *5*).

The results show that when all the amino nitrogens of LPEI are substitued with propyl groups as it is the case with the LPNPEI series, the transfection efficiency is strongly reduced compared to that of the LPEI of 22 kDa (Table 4 and Figure 7). The toxicity of the polymer/DNA complexes on HepG2 cells following transfection was evaluated by measuring the total amount of protein per well. The results indicate that the lower transfection efficiency of LPNPEIs when compared to PEIs is at least partially due to an increased cytotoxicity (Figure 7).

It should be noted that the transfection activity increased with the molar mass of the homopolymers. Interestingly, copolymers of PEtOXZ and LPNPEI are more efficient and less cytotoxic than LPNPEIs. The highest efficiency was obtained with copolymers having

the same or more EtOXZ than NPEI units. These results suggest that other parameters than density of amino nitrogens and buffer capacity are important for gene transfer. It is possible that EtOXZ ensures a higher solubility and/or flexibility of the polymer.

In contrast to the results obtained with LP(EtOXZ-*co*-NPEI) polymers, the most efficient LP(EtOXZ-*co*-EI) copolymers were those having more EI than EtOXZ units. In fact, the most efficient copolymer tested is the one which has a EI fraction of 75%. This proportion has been already found by Jeong and co-workers to be the most efficient on 3T3 cells among a series of random copolymers (*16*).

A comparison of the activity of LP($EtOXZ_{120}$-*co*-EI_{380}) and LP(EI_{380}-*co*-$NPEI_{120}$) showed that the substitution of EtOXZ units by NPEIs induced a decrease of 20-fold of the level of luciferase expression, suggesting again that other parameters than the density in amino nitrogens are important. Among the two polymers containing EtOXZ, EI, and NPEI units, the most efficient was the one having the highest ratio of EI. This latter unit is thus the most important for efficient gene transfer. Of note, the activity of LP($EtOXZ_{20}$-*co*-EI_{270}-*co*-$NPEI_{210}$) is comparable to that of the copolymer LP($EtOXZ_{200}$-*co*-EI_{300}). This can probably be assigned to the fact that they have in common approximately the same fraction of ethylenimine units (about 55%), which corresponds to the fraction of secondary amines of the branched PEI of 25 kDa.

Finally, Bieber and Elsässer recently showed that branched PEIs of low molar weight (LMW) are more efficient in vitro than branched PEIs having high MWs (*18*). We therefore asked whether linear polymers having low molar weights are more efficient than the $LPEI_{22\ kDa}$. Thus, LPEIs of 3.6 and 7.3 kDa were synthesized and their transfection efficiency subsequently evaluated. The results show that the LMW LPEIs allowed expression of significant levels of luciferase, although lower than those obtained by the $LPEI_{22\ kDa}$ (Table 4). Of note, however, the LPEI of 3.6 kDa was as efficient as the branched PEI of 25 kDa (not shown).

These results show that, as it was the case for the LPNPEIs, the transfection activity increased with the molar weight of the LPEI.

In conclusion, this work shows that it is possible to include a fraction of EtOXZ units, which are not positively charged, without decreasing importantly the activity of linear PEIs. We also demonstrate that it is possible to synthesize linear copolymers having tertiary and secondary aziridine units. Polymers containing tertiary amines such as LPNPEIs were significantly less efficient in mediating gene transfer than PEIs, although these polymers contain more amino nitrogens which are not protonated at neutral pH. This result indicates that the capacity to buffer endo-lysosomes is not sufficient to make a molecule an efficient transfection agent. A similar finding was made by Tang and Szoka with starburst dendrimers (*7*, *19*). They found that in contrast to fractured dendrimers, intact dendrimers are not very efficient in gene transfer although they present groups that can be protonated during acidification of endosomes. Taken together, our results show that the high transfection efficiency of PEIs is not solely based on their capacity to buffer endosomes. Other parameters such as flexibility of the polymer may be of importance.

ACKNOWLEDGMENT

Dr. L. Auvray is gratefully acknowledged for neutron scattering analysis. The biological part of this work was performed with the financial support of the Association Française contre les Myopathies (AFM).

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BC0200529